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Probing the Structural Domains and Function *in Vivo* of *Escherichia coli* DNA Topoisomerase I by Mutagenesis

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Insertion and deletion mutagenesis within the gene *topA* of *Escherichia coli* encoding DNA topoisomerase I was carried out to test the existence of subdomains in the enzyme and the relationship between the slow-growth *topA*$^-$ phenotype and the known DNA relaxation activity of the enzyme. All mutants that show no detectable DNA relaxation activity in cell extracts fail to complement the temperature-sensitive growth defect of strain AS17 *topA*$_{am}$ harboring a plasmid-borne temperature-sensitive suppressor tRNA. All mutants that show partial or full levels of DNA relaxation activity in cell extracts (relative to activity in extracts of wild-type cells) can complement this defect. The carboxyl-proximal 25% of the enzyme appears to be in a domain that is dispensable both in terms of the catalytic function of the enzyme and its biological role. Analysis of the mutant enzyme also indicates that the formation of the covalent topoisomerase–DNA complex is correlated with the DNA relaxation activity, which supports the notion that the covalent complex is an obligatory intermediate in the catalysis of DNA topoisomerization.

1. Introduction

Escherichia coli DNA topoisomerase I, a type I enzyme that transiently breaks one DNA strand at a time to affect DNA strand passage, is a relatively large protein containing 863 amino acids (see the preceding paper, Tse-Dinh & Wang, 1986). The determination of the sequence of the gene *topA* encoding this enzyme has made it feasible to test genetically for the existence of domains within it. Here we describe our application of two strategies in this respect. In one, a "linker" DNA oligomer, with a length of an integral multiple of three base-pairs, is inserted at various positions along the gene. This generates a set of in-frame insertion mutants, in which the position of the insertions can easily be mapped by selecting a particular linker sequence containing an appropriate restriction site. This strategy is convenient for introducing mutations, which may or may not have a phenotype, along a structural gene, and has been applied recently in a number of studies (Daubert *et al.*, 1983; Stone *et al.*, 1984; Lobel & Goff, 1984; Baramy, 1985). In a second approach, a short DNA fragment containing a detectable marker, a 21 bp† *lac* operator, and at least one termination codon in each of the three reading frames in either strand, is placed along the gene to give proteins missing various portions of the carboxyl end of the wild-type enzyme.

The construction of these mutants has also enabled us to test the correlation between the catalytic activity of the enzyme and its stoichiometric cleavage of the DNA to give a covalent DNA–protein complex, and between the catalytic activity of the enzyme and its biological function.

2. Materials and Methods

(a) *Bacterial strains and plasmids*

NK7048 (Δ(*lac pro*)$_{XIII}$ *ara nalA argE*$_{am}$ *thi rif*^{R} F′ *lacI*^{q1}) was from N. Kleckner. DM700 (Δ(*cysB topA*)) and MM294 (*thi endA hsr*$_k$) have been described (Wang & Becherer, 1983). AS17 (*topA*$_{am}$ pLL1(Tc^R *supD*^{ts})) was generously provided by Dr R. E. Depew (Northeastern Ohio Universities).

The plasmid pJW312, in which the coding sequence of *topA* is under the control of a wild-type *lac* promoter, was constructed as follows. A 207 bp *Eco*RI fragment containing the *lac* promoter was kindly provided by Dr W. McClure (Carnegie-Mellon University). We have subcloned a region of this fragment from position −81 to +36 in between an *Hha*I site and an *Alu*I site (with position +1 being the major start of the message) as a *Bgl*II fragment in a promoter selector plasmid pJW261 (Wang & Becherer, 1983). In this clone, there is an *Eco*RI

† Abbreviations used: bp, base-pair(s); kb, 10^3 bases or base-pairs; SDS, sodium dodecyl sulfate. Sequence hyphens are omitted throughout this paper.

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site next to the *Bgl*II site near −81. A fragment from this *Eco*RI to an *Hpa*II site within the promoter was isolated. *Bam*HI linkers were ligated to a 95 bp *Alu*I fragment from the 207 bp *Eco*RI fragment containing the *lac* promoter, and the *Hpa*II to *Bam*HI fragment, wherein the downstream part of the promoter resides, was isolated. The two isolated halves of the promoter were then inserted in tandem between the *Eco*RI and *Bam*HI sites of pJW231 (Wang & Becherer, 1983), yielding a 2·4 kb plasmid in which the −81 to +36 region of the *lac* promoter is in between a *Bgl*II and *Bam*HI site, with a *Hin*dIII site downstream from the *Bam*HI site. Finally, a *Bgl*II to *Hin*dIII fragment containing the entire coding sequencing of *topA* was obtained from the plasmid shown in Fig. 2 of Wang *et al.* (1983), and inserted in between the *Bam*HI and *Hin*dIII site of the *lac* wild-type promoter clone. The gene *topA* is thus placed under the control of the *lac* promoter. A diagram of the plasmid is shown in Fig. 1 in Results. For the construction of mutants by insertion of *Hin*dIII linkers, the *Hin*dIII site in pJW312 was deleted. The plasmid was first digested with *Hin*dIII, repairing the ends with *E. coli* DNA polymerase 1, and religation of the repaired ends gave pJW312 Δ*Hin*d.

In order to suppress high levels of *topA* expression from the *lac* promoter, an 1100 bp *Eco*RI fragment containing the entire *lac* repressor gene with an i^q promoter up-mutation was constructed through several cycles of subcloning from pMC7, which was kindly provided by D. M. Calos (Stanford University). The boundary of this fragment proximal to the i^q promoter was originally defined by partial digestion with *Alu*I to cut at a site approximately 250 bp upstream from the *Hin*cII site in the *I* gene. The other boundary was originally defined by *Bal*31 nuclease resection from the *Pst*I site downstream from the termination codon; approximately 600 bp were trimmed off from the *Pst*I site. *Eco*RI linkers were introduced in these positions during the cycles of subcloning. The *I* gene fragment was cloned into pMK16, which carries kanamycin resistance (Kahn *et al.*, 1979), to give pMK16-*lacI*^{q}. This plasmid was used to transform strains AS17 and DM700 to give the host cells used in the screening of the pJW312 plasmids.

(b) *Methods*

Restriction digests, ligation, DNA precipitations with ethanol, agarose gel electrophoresis, polyacrylamide gel electrophoresis, bacterial growth and bacterial transformations were done as described by Maniatis *et al.* (1982). Crude plasmid preparations were obtained according to Ish-Horowicz & Burke (1981). Large-scale plasmid preparations were done according to Garger *et al.* (1983) with minor modifications. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out according to Laemmli (1970), and silver staining of the gel was according to Merril *et al.* (1981). Immunoprecipitations followed basically the procedure of Kessler (1976) except that Brij-58 was used in place of NP40, and two preliminary washes were done in 10 mM-Tris·HCl (pH 7·5), 5 mM-Na_2EDTA, 500 mM-NaCl, 500 μg bovine serum albumin/ml. Western blots were obtained as described by Towbin *et al.* (1979), with the addition of 0·01% (w/v) sodium dodecyl sulfate to their transfer buffer, as suggested to us by Dr T.-S. Hsieh (Duke University).

(c) *Linker insertion*

pJW312 was methylated with *Eco*RI methylase to protect the *Eco*RI sites from cleavage by the restriction enzyme. Four portions of the methylated DNA were then digested separately with *Fnu*DII, *Alu*I, *Hae*III or *Rsa*I, to the extent that 10 to 20% of the circular DNA was converted to full-length linear. The DNA was extracted with phenol, precipitated with ethanol, washed, resuspended, and dodecameric *Eco*RI linkers (CCCGAATTCGGG) were ligated onto the flush ends generated by each restriction enzyme. Excess linkers were removed by precipitation of the DNA with ethanol in the presence of ammonium acetate (the DNA solution was adjusted to 2·5 M-ammonium acetate and 2 vol. ethanol were added) and then digested with *Eco*RI after washing, drying, and resuspension of the precipitate. The DNA was purified by electrophoresis in a 4% polyacrylamide gel. Following electroelution from the appropriate gel slice, the DNA was again precipitated with ethanol, resuspended, digested with *Eco*RI to ensure the removal of multiple copies of linkers ligated to the DNA ends, and purified further by another cycle of gel electrophoresis. The resulting full-length linear fragment was ligated at a concentration lower than 20 μg/ml to enhance the formation of monomeric circles, and transformed into MM294 or NK7048 for screening by restriction analysis. *Hin*dIII linker (CCCAAGCTTGGG) insertion was performed, as described above, except that the *Eco*RI methylation was omitted, and the plasmid pJW312 Δ*Hin*d was used instead of pJW312. The first DNA purification by polyacrylamide gel electrophoresis was also replaced by 2 precipitations with ammonium acetate/ethanol.

(d) *Construction of mutants with premature translation termination*

A 150 bp *Eco*RI restriction fragment, which has translation stop codons in all frames in both directions and a *lac* operator sequence, was previously constructed for mapping the approximate coding regions of genes (S. L. Swanberg & J. C. Wang, unpublished results). The *Eco*RI ends were filled in with the Klenow fragment of DNA polymerase I and *Hin*dIII linkers were ligated on. Following digestion with *Hin*dIII, the fragment was inserted into the *Hin*dIII site of pTR262, an insertion selector plasmid (Roberts *et al.*, 1980). The translation stop fragment, now with *Hin*dIII ends, was prepared and inserted into the *Hin*dIII sites of 4 linker insertion mutants: *topA*1684, *topA*2009, *topA*2277 and *topA*2438. The plasmids with the inserted stop fragment were screened for by transforming the ligation mixture into MM294 (*lac* wild-type) and picking blue colonies on X-gal plates. These transformants must have a *lac* operator site on a multi-copy plasmid. Lysates of the clones were examined for the presence of plasmids in which the translation stop fragment was oriented in the same way.

(e) *Topoisomerase I relaxation assay*

DNA topoisomerase I relaxation activity was assayed by the ability of a crude lysate to relax supercoiled phage PM2 DNA or pJW200 DNA (Wang & Becherer, 1983). The lysis procedure is a modification of the Brij/lysozyme method of Godson & Sinsheimer (1967). The plasmid clones to be examined were transformed into DM700 (Δ*topA*) containing pMK16-*lacI*^{q}. Cells were grown in Luria broth (LB) to an optical density of 1·0. Isopropyl-1- thio-β-D-galactoside (IPTG) was added to 40 μg/ml for at least 40 min near the end of the growth period. One ml of cells was pelleted for 20 s in an Eppendorf centrifuge at 15,000 *g*. The supernatant was removed and the cells

were resuspended in 20 μl of 25% (w/v) sucrose, 40 mM-Tris·HCl (pH 8·0), 1 mM-Na_2EDTA, 1 mg lysozyme/ml, and kept on ice. Twenty μl of 0·9% Brij-58, 10 mM-Tris·HCl (pH 8·0), 60 mM-$MgCl_2$ was then added, and the solution was mixed gently. After 5 min on ice, the debris was pelleted for 2 to 4 min at 4°C at 15,000 *g*. The supernatant was frozen rapidly in solid CO_2/ethanol or liquid N_2 and stored at −70°C, or assayed immediately. Three μl of thawed or fresh supernatant, or appropriate dilutions thereof, were added to 17 μl of assay buffer containing 40 mM-Tris·HCl (pH 8·0), 40 mM-NaCl, 5 mM-$MgSO_4$, 1 mM-Na_2EDTA, 50 μg bovine serum albumin/ml, and 17 μg supercoiled plasmid DNA/ml. Crude supernatants were diluted in assay buffer without DNA, if so indicated. The assay mixtures were incubated at 37°C for 15 min and then adjusted to 0·1% sodium dodecyl sulfate, 2% (v/v) Ficoll-400, 0·2% (w/v) bromophenol blue and loaded on a 0·7% (w/v) horizontal agarose gel. This was run at 1 to 5 V/cm for an appropriate length of time. Staining and photography were as described by Maniatis *et al.* (1982).

(f) *Topoisomerase I cleavage reaction assay*

The same extracts used for assays of relaxation activity (see above) were used for cleavage reaction assays. Plasmid DNA was ^{32}P-labeled by nick-translation as described by Maniatis *et al.* (1982) to a spec. act. of about 10^8 disints/min per μg. DNA in 10 mM-Tris·HCl (pH 8·0) was denatured by heating at 100°C for 1 to 3 min, then quickly cooling to 0°C. Three μl of DNA (5×10^5 cts/min) was added to 5 μl of 20 mM-Tris·HCl (pH 8·0), 20 mM-NaCl, 50 μg bovine serum albumin/ml. Then 2 μl of crude extract was added. The mixture was incubated at 37°C for 30 min. Five μl of 150 mM-NaOH was then added, and the incubation at 37°C was continued for about 1 min. The NaOH was neutralized by adding 1·5 μl of an equal volume mixture of 1 M-HCl and 1 M-Tris·HCl (pH 8·0). $CaCl_2$ was added to 2 mM and micrococcal nuclease was added to 20 units/ml ("μM" units) and the mixture was incubated at 37°C for 20 min. Nine μl of 3× Laemmli sample buffer was added and the samples were run on a 7% gel (Laemmli, 1970). Gels were then dried and autoradiographed as described by Maniatis *et al.* (1982).

(g) *Complementation of a chromosomal* $topA_{am}$

Strain AS17 is a $topA_{am}$ strain bearing a plasmid-borne temperature-sensitive suppressor *supI*[ts]. It grows at 30°C but not at 42°C. Plasmids with mutant *topA* genes to be tested were transformed into AS17 containing a pMK16-*lacI*[q] plasmid, and transformants were tested for their ability to grow at 30°C and 42°C. Both low- and high-copy number $topA^+$ plasmids permit growth at 42°C.

3. Results

(a) topA *in-frame insertion mutants*

A set of 20 in-frame insertion mutants was constructed by the insertion of a dodecameric *Eco*RI (CCCGAATTCGGG) or *Hind*III (CCCAAGCTTGGG) linker into different positions along a plasmid-borne *topA* gene of *E. coli* (Fig. 1). The sites of insertion were generated by digesting the DNA lightly with one of the restriction enzymes selected for yielding flush ends; the positions of insertion can therefore be deduced by restriction

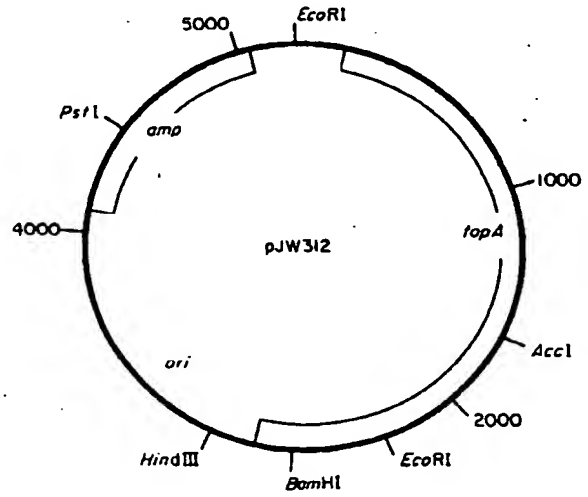

Figure 1. A 5·2 kb plasmid (pJW312) used in the construction of *topA* mutants. The coding region of the *topA* gene is placed downstream from a wild-type *lac* promoter, located to the right of the *Eco*RI site at the top of the Figure, and the direction of transcription of the gene is clockwise. For the insertion of *Hind*III linkers into *topA*, the *Hind*III site in the plasmid was destroyed to give pJW312 Δ*Hind*III; see the text for details of the construction of these plasmids.

mapping and the known sequence of the *topA* gene (see the preceding paper, Tse-Dinh & Wang, 1986). In most cases, each mutant is numbered by the number of nucleotides between the ATG start of the gene and the particular linker inserted. The mutant *topA*127H, for example, has a *Hind*III linker preceded by 127 bp of the *topA* coding sequence including the ATG start; similarly, the mutant *topA*341R has an *Eco*RI linker preceded by 341 bp of the *topA* gene starting from the first ATG codon. Several tests were carried out to ensure that only a single dodecamer linker had been inserted in each of the mutants. First, the insertion of more than one dodecamer would generate at least one *Apa*I restriction site (GGGCCC), which is otherwise absent in the plasmid. With the exception of mutant *topA*2114H, the clones are resistant to *Apa*I. Examination of the *topA* sequence shows that the inserted linker in *topA*2114H is flanked by three G residues on one side, and therefore the insertion of one copy of the linker into this site is expected to create an *Apa*I recognition sequence. Second, appropriate restriction enzymes were used to examine whether the length of the insert-containing fragment of each clone was longer than the corresponding fragment from the control without an insert by the expected length of 12 bp. Finally, to test the possibility of inadvertent small deletions or additions during cloning, which might give frameshift mutants, electrophoresis of crude cell extracts of the mutants in SDS/polyacrylamide gels was carried out. Immunostaining of protein bands, after their transfer to a nitrocellulose sheet,

with antibodies specific to *E. coli* DNA topoisomerase I ("Western-blotting") shows that the sizes of the mutant enzymes are not significantly different from the wild-type protein. In several cases, DNA topoisomerase I in the crude extracts was immunoprecipitated first and then analyzed by SDS/polyacrylamide gel electrophoresis. In these cases, again, no detectable change of the protein size was observed.

The relative levels of DNA topoisomerase I activity in cell extracts of an *E. coli* Δ*topA* strain carrying different plasmid-borne mutant *topA* genes, described above, were examined according to the procedure described in Materials and Methods. Among the family of 20 mutants, seven have no detectable amount of active DNA topoisomerase I (less than 1% of activity in control extracts of cells carrying the same plasmid without an insert in the *topA* sequence). Of the remaining 13 mutants, extracts of 12 show 20% to full level activity relative to extracts of control cells harboring the *topA*$^+$ plasmid. Extracts of mutant 874H showed variable amounts of activity; the reason for this variability will be described later.

The in-frame insertion mutagenesis results suggest that *E. coli* DNA topoisomerase I is relatively tolerant of insertional perturbations in its primary sequence: at least 12 out of a total of 20 mutants show high levels of activity. The carboxyl-terminal portion of the enzyme appears to be particularly insensitive to insertions. Seven mutants in a 400 bp segment near the 3′ end of the gene (2009H, 2114H, 2185H, 2277H, 2393H, 2406R and 2438H) all yield active enzymes.

(b) topA *mutants missing variable amounts of the 3′-terminal coding sequence*

To test further whether the carboxyl-terminal portion of *E. coli* DNA topoisomerase I is non-essential for its enzymatic activity, we have constructed nonsense mutants to terminate the polypeptide chain prematurely. A short DNA fragment containing a stop codon in each of the reading frames was introduced into the *Hin*dIII site on the linker inserted in mutants 1684H, 2009H, 2277H and 2438H, as described in Materials and Methods, and the resulting mutants were designated 1684ter, 2009ter, . . ., etc.

Sizing of the proteins produced in these nonsense mutants by SDS/polyacrylamide gel electrophoresis shows that the molecular weights of the proteins bearing antigenic determinants of DNA topoisomerase I are 65,000, 79,000, 86,000 and 96,000 M_r, respectively, for mutants 1684ter, 2009ter, 2277ter and 2438ter. The sizes of the proteins are

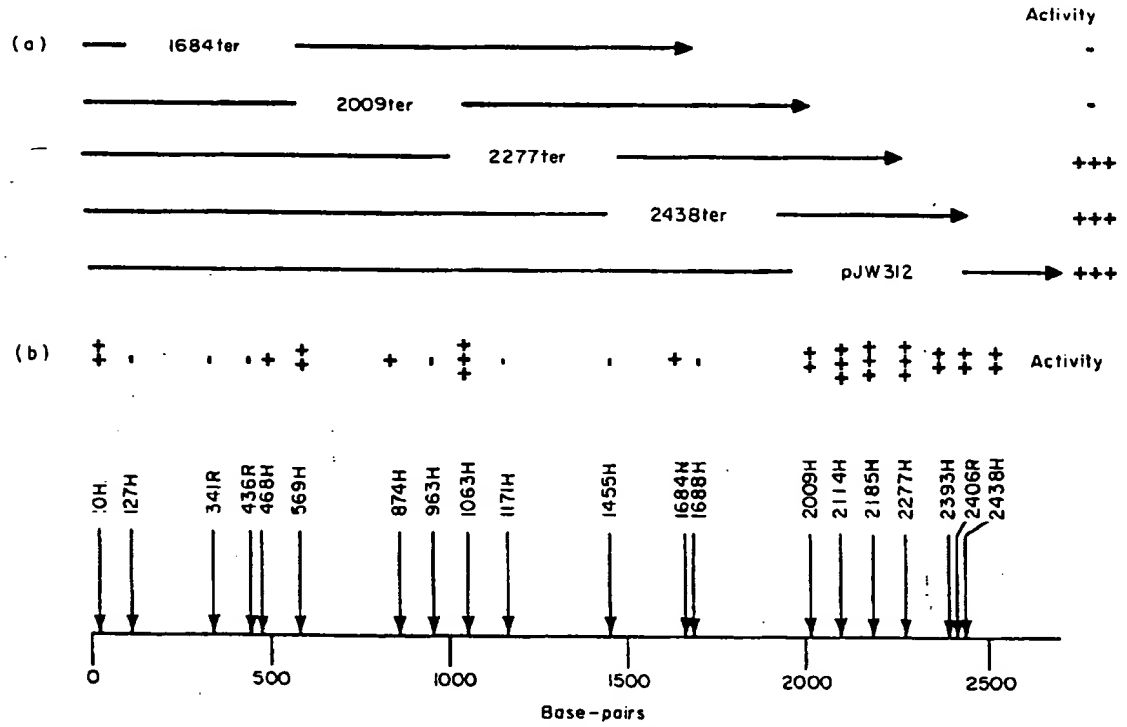

Figure 2. The location of the linker insertion sites, and the extent of translation of the termination mutants. In most cases, each mutant/mutation is numbered with the number of nucleotides between the ATG start of the gene and the particular linker inserted. The mutation *topA*127H for example, has a *Hin*dIII linker preceded by 127 bp of the *topA* coding sequence including the ATG start; similarly, the mutation *topA*341R has an *Eco*RI linker preceded by 341 bp of the *topA* gene starting from the first ATG codon. The abscissa is in base-pairs, with the A of the translation initiation ATG being bp 1. The presence or absence of DNA relaxation activity in lysates is also indicated. Mutant nomenclature and activity levels are as described in the legend to Table 1.

thus in agreement with those expected from translational termination within the inserts. There is no detectable topoisomerase I activity in cell extracts of 1684ter and 2009ter, whereas in extracts of 2277ter and 2438ter the levels are comparable to that of the wild-type control. Because the amounts of topoisomerase I in cell extracts of these strains are comparable, the differences in the levels of DNA topoisomerase I activity reflect differences in specific activities. Thus, the removal of 11% of the protein from its carboxyl terminus has little effect on its DNA topoisomerization activity. Figure 2 summarizes the positions of the insertion and deletion mutations and the enzymatic activities of the mutants.

(c) *Covalent complex formation between mutant DNA topoisomerase I and DNA*

We have examined the ability of mutant DNA topoisomerase I in extracts of cells to cleave DNA and link covalently to it. Cell extracts from different mutants were incubated with denatured ^{32}P-labeled DNA, and NaOH was added to the mixture to effect the formation of the covalent complex. Following neutralization and extensive digestion of the mixture with staphylococcal nuclease, SDS/polyacrylamide gel electrophoresis of the mixture was carried out (see Materials and Methods for details). The relative amounts of ^{32}P associated with the topoisomerase I mutant proteins were assessed by examining an autoradiograph of the gel. These results are shown in Table 1. In general, the ability of an *E. coli* DNA topoisomerase I mutant protein to effect DNA cleavage and form a covalent complex with it upon treatment with a protein denaturant, alkali in the present case, correlates with its DNA topoisomerization activity assayed by the relaxation of negatively supercoiled DNA. The seven mutants that did not give detectable amounts of DNA topoisomerase I relaxation activity in extracts yielded no detectable amounts of the covalent complex. Mutant 2393H might be of interest. Although the relaxation activity in cell extracts of this mutant appears to be significantly lower than that of the wild-type control, the amount of the covalent complex formed with mutant extracts seems at least as high as that with the control.

(d) *Correlation between the relative activity of DNA topoisomerase I* in vitro *and its physiological role* in vivo

It has been shown that a tight *topA* mutation can not be transduced readily into *E. coli* except in

Table 1

Characteristics of the dodecameric linker insertion mutations

Insertion site[a]	Mutation[b]	Relaxation activity[c]	Complement *topA*$^-$	Covalent complex formation[d]	Antigen levels[d]	Restriction site[e]
1327	10H	+ +	+	+	ND	A
1444	127H	−	−	−	+ +	A
1658	341R	−	−	−	+ +	F
1753	436R	−	−	−	+ +	F
1785	468H	+	+	+	ND	A
1886	569H	+ +	+	+ +	ND	H
2191	874H[f]	+	+	−	+	A
2280	963H	−	−	−	+ +	R
2380	1063H	+ + +	+	+ +	ND	R
2488	1171H	−	−	−	+	R
2772	1455H	−	−	−	+ +	A
3001	1684H	+	+	+	+ +	A
3005	1688H	−	−	−	+ +	R
3326	2009H	+ +	+	+ +	+	A
3431	2114H	+ + +	+	+ +	ND	H
3502	2185H	+ + +	+	ND	ND	H
3594	2277H	+ + +	+	+ +	ND	A
3711	2393H	+ +	+	+ + +	+ +	A
3725	2406R	+ +	+	+ +	+ +	F
3757	2438H	+ +	+	ND	ND	H
None	pJW312	+ + +	+	+ +	+ +	−

See the text for descriptions of activity assays and complementation of the temperature sensitivity of AS17.

[a] Sites of linker insertion according to the sequence reported by Tse-Dinh & Wang (1986).

[b] With the exception of 2393H, 2406R and 2438H, numbering was from the initiation codon, A of ATG = 1.

[c] For relaxation activity levels: −, none detectable; +, low levels (<20% of levels of pJW312); + +, intermediate levels (20 to 75%); + + +, wild-type levels (>75% of pJW312).

[d] For the formation of the covalent complex and antigen levels; −, none detected; +, intermediate levels; + +, wild-type levels; + + +, greater than wild-type levels; ND, not determined.

[e] The restriction site into which the dodecameric linker was inserted: A, *Alu*I; F, *Fnu*DII; H, *Hae*III; R, *Rsa*I.

[f] See the text for a description of the unusual aspects of this mutant.

strains carrying compensatory mutations in certain genes (DiNardo *et al.*, 1982; Pruss *et al.*, 1982; Laufer & Depew, 1984; Raji *et al.*, 1985). This suggests that the enzyme normally serves an important role, but physiological adjustments can occur to maintain cell viability in the absence of the enzyme. Further evidence supporting a crucial cellular role of the enzyme came from the construction of two *topA* conditional mutants in which compensatory mutations are absent. One of the conditional *topA* mutants contains the coding sequence of *topA* expressed from *lac* promoter, and it has been shown that the repression of the promoter strongly retards the growth of this strain (Kirkegaard *et al.*, 1984; Wang, 1984). In the other strain, a *topA* amber mutation is suppressed by a plasmid-borne temperature-sensitive suppressor tRNA. This strain, AS17 $topA_{am}$ pLL1 ($supD^{ts}$), grows at 30°C but not 42°C (Stamkiewicz & Depew, 1983).

The construction of a large number of mutants expressing active and inactive DNA topoisomerase I in the present work makes it possible to test further whether the DNA relaxation activity of the enzyme correlates with its physiological function. Plasmids carrying in-frame insertion or nonsense mutations in *topA* were used to transform strain AS17, and the ability of the plasmids to complement the growth defect of the strain at non-permissive temperatures was examined. As shown in Table 1, a strong correlation is found between the absence of active DNA topoisomerase I in cell extracts and the growth defect. Seven in-frame insertion mutants and two nonsense mutants that show no detectable enzyme activity in cell extracts do not complement the growth defect of AS17 as its non-permissive temperature. On the other hand, AS17 transformed with plasmids which produce substantial amounts of active DNA topoisomerase I can grow at temperatures that inactivate the chromosomally coded enzyme.

(e) *Reversion of mutant* topA*874H*

As mentioned earlier, assays of DNA topoisomerase I activity in mutant 874H yielded variable results. Different experiments gave levels ranging from less than 1% of the $topA^+$ control to 20%. It turns out that this variability is due to the reversion of the mutant to $topA^+$.

The *topA* temperature-sensitive strain AS17 $topA_{am}$ pLL1 ($supD_{ts}$) was transformed with the plasmid bearing *topA*874H, and the growth of the transformants at 30°C and 42°C was compared with control cells transformed with pJW200, a PBR322 derivative in which sequences between the *Hae*II sites at locations 232 and 2349 of pBR322 were deleted (Wang & Becherer, 1983). At 42°C, the plating efficiency of AS17 pLL1 harboring pJW200 on LB-agar dishes was less than 10^{-4}, and no colonies were visible after 48 hours of incubation; the same strain transformed with a $topA^+$ plasmid showed a plating efficiency of one, and gave good sized colonies (1 to 2 mm in diameter) after 24 hours. AS17 pLL1 transformed with *topA*874H gave no visible colonies after 24 hours; colonies of heterogeneous sizes appeared, however, at a frequency of about 10^{-2} after 48 hours. These colonies, upon restreaking on agar plates, grew well at 42°C and gave colonies of fairly uniform size. Extracts of cells grown at 42°C from a dozen randomly picked colonies were assayed for DNA relaxation activity, and all were positive. Five of these extracts were assayed further in the presence and absence of rabbit antibodies raised against *E. coli* DNA topoisomerase I, and the DNA relaxation activity in all five was inhibited by the antibodies. These results show that transformants that can grow at 42°C have acquired active DNA topoisomerase I.

To determine whether the chromosomal copy of $topA_{am}$ in strain AS17 or the plasmid-borne copy of *topA*874H has reverted, plasmid DNA samples were prepared from the dozen $topA^+$ colonies picked and used to transform strain DM700 (Δ*topA*). One transformant was selected from each transformation. Cell extracts of these transformants were assayed for DNA relaxation activity, and plasmid DNAs recovered from these transformants were examined by restriction mapping. None of the dozen recovered plasmids show gross rearrangements. Seven of the transformants show DNA relaxation activity in cell extracts. Because the entire chromosomal *topA* gene is deleted in DM700, the presence of topoisomerase I activity shows that the plasmid-borne *topA*874H has reverted. Of these

Table 2
Characteristics of the C-terminal deletion mutations

Insertion site	Mutation	Relaxation activity	Covalent complex	Complement $topA^-$	Antigen level	Predicted size ($\times 10^{-3}$ M_r)	Measured size ($\times 10^{-3}$ M_r)
3001	1684ter	−	−	−	++	65	65–72
3326	2009ter	−	−	−	++	78	79
3594	2277ter	+++	+++	+	++	87	86
3757	2438ter	+++	++	+	+++	94	96
None	pJW312	+++	++	+	++	99	100

The insertion site, mutant numbering, activity, and antigen levels are as described for Table 1. The predicted molecular weights were determined by translating the modified DNA sequence. Measured sizes were calculated from electrophoretic mobilities in SDS/polyacrylamide gels.

seven transformants, the plasmid DNA of three had lost the *Hin*dIII site inserted; plasmid DNAs recovered from the other four are all tandem dimers, each with one copy of the *topA* gene containing a *Hin*dIII site and the other copy of the *topA* gene without a *Hin*dIII site. The five transformants that show no DNA relaxation activity in their extracts all yielded plasmids with a *Hin*dIII site at the expected location.

The simplest interpretation of these results is that *topA*874H can readily revert to *topA*$^+$ by modifications at or near the inserted *Hin*dIII linker. Because of the multi-copy nature of the plasmid, transformants of AS17, which become temperature-insensitive, may harbor a mixture of the original *topA*874H plasmid and its *topA*$^+$ revertants.

4. Discussion

Our data indicate that the activity of *E. coli* DNA topoisomerase I is fairly tolerant of the insertion of four amino acids at different positions along the polypeptide chain, particularly in the carboxyl-terminal region of the enzyme. Of the 20 insertion mutants, 12 have retained partial to full level activity, and seven of these 12 have inserts within the carboxyl-proximal 25% of the protein. The non-essentiality of the carboxyl-terminal portion of the enzyme was confirmed further by deletion studies: at least the carboxyl-proximal 11% of the protein can be deleted without loss of activity. These results suggest that the carboxyl-terminal portion of the enzyme is in a domain that is structurally and functionally non-essential. The mutant *topA*2009ter, in which the carboxyl-proximal 22% of the enzyme is deleted, yields an inactive protein. The deletion strategy employed substitutes the deleted sequence with a stretch of amino acids (see Materials and Methods), however, and it might be the substitution rather than the deletion *per se*, which inactivates the enzyme. It is plausible that the entire carboxyl-proximal 25% of the protein, which contains the cluster of seven non-inactivating inserts, is in a dispensable domain.

Biochemical and genetic characterization of the DNA topoisomerase I mutants show that the relaxation activity and the physiological role of the enzyme are strongly correlated. Plasmids containing mutated *topA* genes can complement AS17 *topA*$_{am}$ pLL1 (*supD*^{ts}) at a non-permissive temperature if the mutant topoisomerase shows DNA relaxation activity *in vitro*, and fail the complementation test if the mutant enzyme shows no detectable relaxation activity *in vitro*. The mutant *topA*874H is particularly interesting in that its complementation of the temperature sensitivity of strain AS17 is correlated with the reversion of the mutant to give active DNA topoisomerase I.

Our studies of the mutants also indicate that the formation of the covalent protein–DNA complex is correlated with the relaxation activity, which supports the notion that the covalent complex is an obligatory intermediate in the relaxation reaction (Wang, 1971). It is plausible, however, that a class of mutants exists that can form the covalent complex, but is blocked in the DNA rejoining step. In this context, the mutant *topA*2393H enzyme might be one that forms the covalent complex readily.

Table 3
Peptide inserted into mutant proteins

Peptide inserted	Mutation	Relaxation activity
Ala-Gln-Ala-Trp	10H	+ +
	127H	−
	874H	+
	1684H	+
	2185H	+ + +
Ala-Arg-Ile-Arg	2438H	+ +
Pro-Asn-Ser-Gly	436R	−
	341R	−
Pro-Lys-Leu-Gly	2406R	+ +
	468H	+
	963H	−
	1456H	−
Pro-Ser-Leu-Gly	2277H	+ + +
	569H	+ +
	1688H	−
	2009H	+ +
	2114H	+ + +
	2393H	+ +
Ser-Gln-Ala-Trp-Asp†	1063H	+ + +
	1171H	−

Mutant numbering and activity levels are described in the legend to table 1.

† Accompanied by the loss of a Tyr, for a net gain of 4 amino acids.

As a method of obtaining in-frame insertion mutants, linker insertion at restriction sites has the advantage that the positions of mutation and the nature of sequence changes can be rapidly determined. Because the insertion sites are generated by restriction enzymes, the mutants obtainable are not completely random, both with respect to their positions in the gene and the nature of the amino acid changes. The insertions into the *Hae*III sites (GGCC), for example, disrupt a glycine codon in three of the four sites. The changes in amino acid sequence resulting from linker insertion depend on both the linker sequence and the sequence of the insertion site; three of the four restriction enzymes we used to give flush-ends cut in between a G and a C residue, and as a consequence there are only six different amino acid sequence inserts among the 20 mutants (see Table 3). There does not appear to be any obvious activity level bias due to the specific amino acid sequence inserted. The seven well-tolerated inserts in the carboxyl-terminal region are not restricted to a particular class of amino acid sequence insertions, nor do they seem to be excluded from any of the sequence classes. Conversely, the inserts that inactivate topoisomerase I do not seem to be restricted to, nor excluded from, any of the sequence classes.

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